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Original Article

Tanshinone IIA pretreatment attenuates oxygen-glucose deprivation induced hippocampal neurons damage

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Abstract: Objective: Tanshinone IIA (Tan IIA) has been used in treatment of ischemic stroke in China. This study was designed to investigate the underlying neuroprotective mechanism of Tan IIA pretreatment in primary cultured rat hippocampal neurons incurred oxygen-glucose deprivation (OGD). Method: Hippocampal neurons was prepared from E18 embryonic rats and identified by immunocytochemical staining. After OGD and pretreatment with Tan IIA, cell viability was measured using MTT assay. Lactate dehydrogenase (LDH) activity, Caspase-3 activity and adenosine triphosphate (ATP) level were detected using assay kits. Cell apoptosis analysis was carried out with flow cytometry. Mitochondrial membrane potential and intracellular calcium were monitored by fluorospectrophotometer. Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 (UQCRFS1) expression was mesured by RT-PCR and Western blotting. Results: Hippocampal neuron were incurred OGD for 6 h, the cell survival was decreased to (61.5 ± 4.1)% comparing to the control group (set 100%). Pretreatment with Tan IIA at 3, 10, 30 and 100 mM, the cell survivals were (70.5 ± 2.1)%, (72.8 ± 4.4)%, (78.8 ± 3.4)% and (83.5 ± 3.1)% respectively and higher than that of only OGD-ed group. Exposure of the cells to OGD, cell apoptosis was induced, intercellular Caspase-3 activity was elevated, ATP level was decreased and LDH activity was increased in the culture supernatants. Pretreatment with Tan IIA alleviated all the changes. Furthermore, OGD may induce mitochondrial membrane potential decreasing and intracellular calcium level elevating in the neuron, Tan IIA pretreatment also alleviated these effects. When the cells were exposed to OGD, both mRNA and protein level of UQCRFS1 were decreased, Tan IIA pretreatment attenuated the changes caused by OGD. Conclusion: Tan IIA pretreatment can reduce energy damage of the neurons induced by OGD, which is associated with attenuating the changes in mitochondrial membrane potential and UQCRFS1 expression.

Keywords: Hippocampal neurons, oxygen-glucose deprivation, Tanshinone IIA, Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1

Introduction

Ischemic brain injury is one of the major contributors to mortality and disability worldwide and a major public health problem [1, 2]. It is often caused by a transient or permanent reduction of cerebral occlusions [3] and leads to serious and complex pathophysilogical changes affecting multiple levels of the brain and sports a high global mortality rate as the leading cause of neurological disability [4, 5]. The brain is very susceptible to damage by energy-depriving injuries which can result in membrane depolarization, inflammation and apoptosis [5, 6], due to low energy levels, high aerobic metabolism, and low concentrations of radical-scavenging enzymes [7]. Extensive efforts have been made to develop new chemicals and methods for prevention and treatment of ischmic brain injury. Accumulating evidences from animal studies and clinical trials have shown that ischemic postconditioning, a series of rapid intermittent interruptions of blood flow in the early phase of reperfusion that mechanically alters the hydrodynamics of reperfusion [8], is an effective procedure to suppress secondary tissue injury following recovery of blood supply in brain [9], as well as in heart, liver and intestine [10]. Recent studies have focused especially on ischemic preconditioning, known as induced tolerance to trigger an intrinsic neuroprotective mechanism [11, 12]. Although

ischemic preconditioning is considered as a potential strategy for treating brain ischemic injury, it has never progressed to detailed clinical investigation [13]. This was largely due to the logistics of inducing preconditioning ischemia in brain in advance of a more prolonged insult (e.g., that would lead to stroke or myocardial infarction). The alternative strategies are chemicals, which were shown to be effective/feasible treatments for some brain diseases (or brain ischemic injury) [14, 15].

Tanshinone IIA (Tan IIA) is a key compound purified from the Chinese herb Danshen (Radix Salviae Miltiorrhiza Bge), it has been reported functioning as an antioxidant and anti-inflammatory reagent *in vitro* and *in vivo* [16, 17]. Tan IIA has been commonly used in China for the treatment of angina pectoris and cerebrovascular disorders such as ischemic stroke [18, 19]. Tan IIA can protect rats and mice against cerebral ischemic injury [20]. Recent study showed that Tan IIA has beneficial effects on brain through enhancing neuron regeneration and inducing cell proliferation [21]. Tan IIA pretreatment has also been reported to have neuroprotective effects on neonatal hypoxia-ischemia brain damage [22] and transient or permanent focal cerebral ischemia [23]. Moreover, pretreatment with Tan IIA can prevent the neuroblastoma cell line PC12 from apoptosis induced by serum-free culture conditions [24]. The effects might be associated with its antioxidative potential [22, 25], blocking the activation of HIF-1α/SDF-1 pathway [26] and mediating brain iron homeostasis [20]. These studies suggest that Tan IIA pretreatment has beneficial effects on ischemic brain injury. However, the underlying mechanisms remain unclear. Mitochondrial permeability transition pore plays a role in cardioprotection induced by Tan IIA pretreatment [27]. Currently, no report focus on the effect of Tan IIA pretreatment on mitochondria of brain or neurons. In this study, we analyzed the neuroprotective effects of Tan IIA pretreatment and its effects on mitochondrial membrane potential and energy metabolism of primary rat hippocampal neurons.

Material and methods

Hippocampal neuronal culture

Use of animals has been approved by the Institutional Animal Care and Use Committee of Guilin Medical University. For all experiments, we used an *in vitro* system of primary cultures of hippocampal neurons prepared from E18 embryonic rats (SPF experimental animal center of Guilin Medical University, Guilin, China). Briefly, hippocampi were dissected and removed from embryonic rats and placed in cold phosphate buffered saline solution (PBS) containing penicillin, streptomycin, and amphotericin B. The tissues were stripped of meninges and blood vessels, minced in a thermally insulated container on ice, and dissociated by enzymatic digestion. Cells dissociated from the hippocampi were pooled and resuspended in Dulbecco's minimum essential medium (DMEM) containing 10% F12 medium and 10% fetal bovine serum (FBS) (all from Gibco BRL, Grand Island, NY, USA). The cells were further seeded in poly L-lysine (Sigma, St.Louis, MI)-coated 96-well culture plates (for cell viability assay), 35-mm tissue culture dishes with poly L-lysine-coated 22-mm coverslips (for immunocytochemical staining) and 6-well culture plates (for other experiments) at the density of 1×10^4 per cm^2. The second day, the medium was changed to the medium with 1% serum. The cultured cells were used from *in vitro* days 11 to 13.

Immunocytochemical staining

Immunocytochemistry was performed with a monoclonal anti-rat neuron-specific enolase (NSE, a neuronal marker [28]) antibody (Santa. Cruz, CA) and Elivision™ plus polyer HRP IHC kit (Maixin Biological Technology Ltd., Fujian, China). Briefly, neurons on coverslips were fixed, blocked with blocking reagent and then incubated with NSE antibody (2 μg/ml) at room temperature for 1 hour and washed with PBS. The cells were incubated with polymer enhancer for 20 min, then followed with polymerized HRP-anti mouse/rabbit IgG antibody for 50 min and rinsed in PBS between the incubation steps. After washed with PBS, peroxidase reaction was performed by use of 3, 3'-diaminobenzidine for 5 min. After counterstained with haematoxylin, the cells were dehydrated and mounted. Cells known to show strong immunostaining for NES were used receiving either the primary antibody or PBS as positive and negative controls. In all the staining procedures, the positive controls showed staining clearly and there was no staining in the negative controls.

Cell viability

Cell viability was measured using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MI) assay. The cultured hippocampal neurons in 96-well culture plates were divided into control group [cultured neurons, which were neither pretreated with Tan IIA (the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) nor incurred OGD], OGD group (neurons were only OGD-ed for 6 h) and Tan IIA-pretreated groups (cells were pretreated with stepwise concentrations of Tan IIA for 12 h and then OGD-ed for 6 h). Briefly, the neurons in Tan IIA-pretreated groups were pretreated with stepwise concentrations of Tan IIA for 12 h, then the cells in OGD group and Tan IIA-pretreated groups were OGD-ed for 6 h, the 5 mg/ml MTT solution was added into each well of all groups and incubated at 37°C with 5% CO_2 for 4 h. After the removal of culture medium, 150 μl dimethyl sulfoxide was added into each well to dissolve the formazan. The optical density was measured at 490 nm using an iMARK™ Microplate Reader (Bio-Rad, USA). Cell viability was expressed as the percentage of survival of control group (100%).

Lactate dehydrogenase (LDH) release and adenosine triphosphate (ATP) bioluminescence assay

The release of cytoplasmic LDH to conditioned media was measured with the LDH Activity Kit (Beyotime Institute of Biotechnology, Haimen, China) as an indication of compromised cellular integrity. The assay is based upon a coupled enzymatic conversion from 2-p-iodophenyl-3-nitrophenyl tetrazolium chloride (INT) into a red formazan product. Absorbance data of each well, which is proportional to the number of damaged cells, was read at 490 nm by the microplate reader. The mean absorbance of each group was used to calculate relative LDH level (set control group as 100%).

Following Tan IIA pretreatment, intracellular ATP levels were determined using a bioluminescent ATP assay kit (Beyotime Institute of Biotechnology, Haimen, China) following the manufacturer's protocol. Briefly, the cells were disrupted in 200 μL lysis buffer and centrifuged at 12,000/g to collect the cell supernatant. ATP content was measured using an ATP assay kit. Protein abundance was measured using a Bradford protein assay (Beyotime Institute of Biotechnology, Haimen, China), and ATP abundance was normalized to total protein abundance. ATP level was showed as % of control.

Flow cytometric analysis

Hippocampal neurons were harvested and resuspended with PBS and labeled with Annexin V and PI in Apoptosis Detection kit (BD Pharmingen, Bedford, USA) according to the manufacturer's protocol. Cell apoptosis analysis was carried out with flow cytometry (BD FACSAria, BD Biosciences).

Caspase 3 activity assay

Hippocampal neurons were collected, washed twice with ice cold PBS and lysed in ice-cold cell lysis buffer (25 μl per 1×10^6 cells) for 15 min on ice. After centrifugation at 12,000 g for 2 min, the supernatant was collected for caspase-3 activity determination by Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to manufacturer's protocol. The caspase-3 activities were expressed as the percentage of the control group (%).

Mitochondrial membrane potential (Δψ) studies

The cationic fluorophore Rhodamine 123 (R123) concentrates in energized mitochondria and yields a punctate intracellular fluorescence in proportion to Δψ. Neurons in each group were collected and loaded with R123 (Sigma, St Louis, MO, USA) at 500 nmol/L for 30 min at 37°C and washed with experimental buffer. The fluorescent intensity of cells (5×10^5/ml) was monitored using a dual-excitation fluorospectrophotometer (EL05113056, Cary Eclipse, Australia) at 488 nm excitation and 535 nm emission. The mean fluorescent intensity in each group was calculated and expressed as mean ± SD.

Intracellular calcium ($[Ca^{2+}]_i$) measurement

Changes in $[Ca^{2+}]_i$ were measured by the ratiometric method in fura-2 AM-loaded cells at 340 and 380 nm for excitation and 510 nm for emission ($F_{340, 510}/F_{380, 510}$). This ratio represents

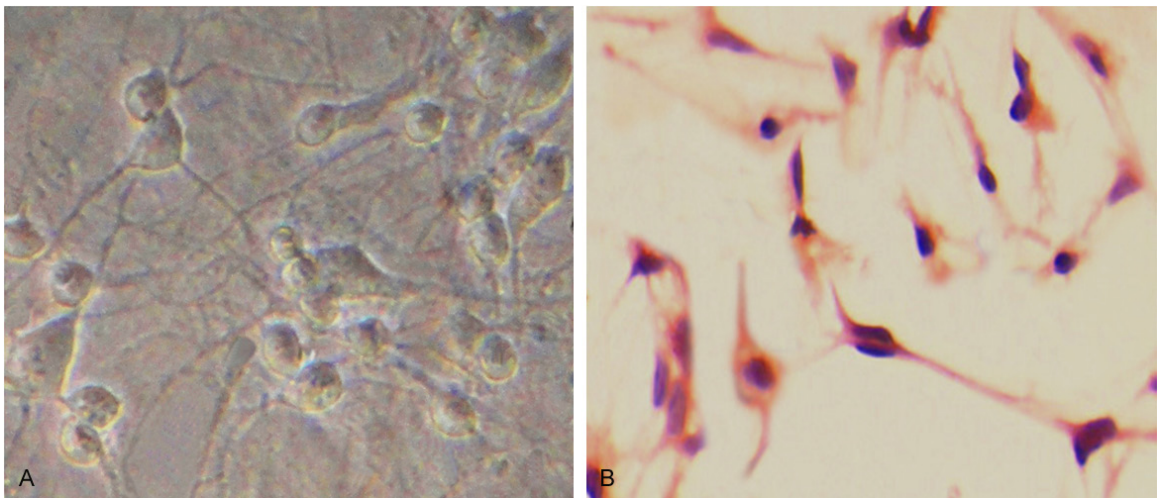

Figure 1. Cultured hippocampal neurons (A) (400×) were characterized by NSE Immunocytochemical staining (B) (400×).

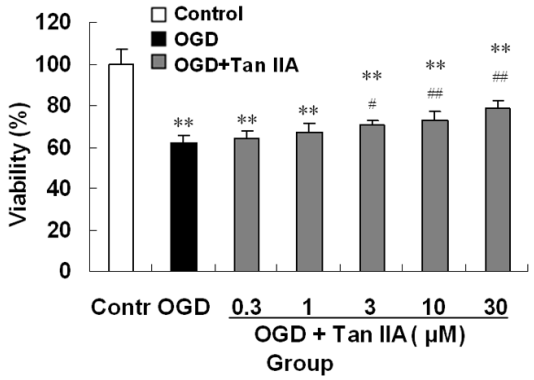

Figure 2. Effects of Tan IIA pretreatment on cell viability of cultured hippocampal neurons incurred OGD. Contr represents control group in which hippocampus neurons were neither pretreated with Tan IIA nor incurred OGD. The result was expressed as mean ± SD from three independent experiments. Calculation of statistical data was done by the statistical computer system GraphPad Instat 3. One-way analysis of variance (ANOVA), unpaired t-test, and the two-tailed *P* value were used. **P<0.01 compared to control; #P<0.05, ##P<0.01 compared to OGD-ed group without Tan IIA pretreatment.

relative changes in $[Ca^{2+}]_i$ [29]. After two washes with a loading buffer consisting of (in mM) 150 NaCl, 5 KCl, 5 glucose, 1 $MgCl_2$, 2.2 $CaCl_2$, and 10 HEPES, pH 7.4, neurons (1×10^6/ml) of each group were incubated with 5 μM fura-2 AM (Sigma, St Louis, MO, USA) and 0.2% bovine serum albumin in the same buffer at 37°C for 30 min in dark. Then, the fura-2 AM was removed by washing. The cells were incubated at 37°C for 10 min to convert the fura-2 AM-ester to the free acid form under the action of nonselective esterase. Cultures were washed twice to remove excessive Fura-2 AM and incubated for an additional 20 min in dark to complete de-esterification of the dye. The fluorescence intensity ratio of the Fluo-2/AM loaded cells was monitored using a dual-excitation fluorospectrophotometer (EL0511-3056, Cary Eclipse, Australia). The results were expressed as means ± SD.

Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 (UQCRFS1) expression

For UQCRFS1 mRNA expression, real-time RT-PCR was carried out. Briefly, Total RNA was extracted from the neurons with Trizol reagent (Invitrogen, Carlsbad, CA). The purity and quantity of RNA were determined with an ultraviolet spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) with A260/A280 ratio >1.8. Extracted RNA was reverse transcribed with Prime Script™ RT reagent kit and oligo-dT primers (TaKaRa Biotechnology Co., Ltd. Dalian, China). The primers for the selected gene (NM_001008888, Position: 662-876, Forward: GAGCGTGTAAAGAAGCCTGAAT, Reverse: CCCACAACAACTACATCACCAC) were designed and synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). The SYBR Green Master Mix (Applied Biosystems, Foster City, CA) was used for real-time RT-PCR analysis. Differences in gene expression were calculated using threshold cycle (Ct) values with the internal control of GAPDH (NM_017008,

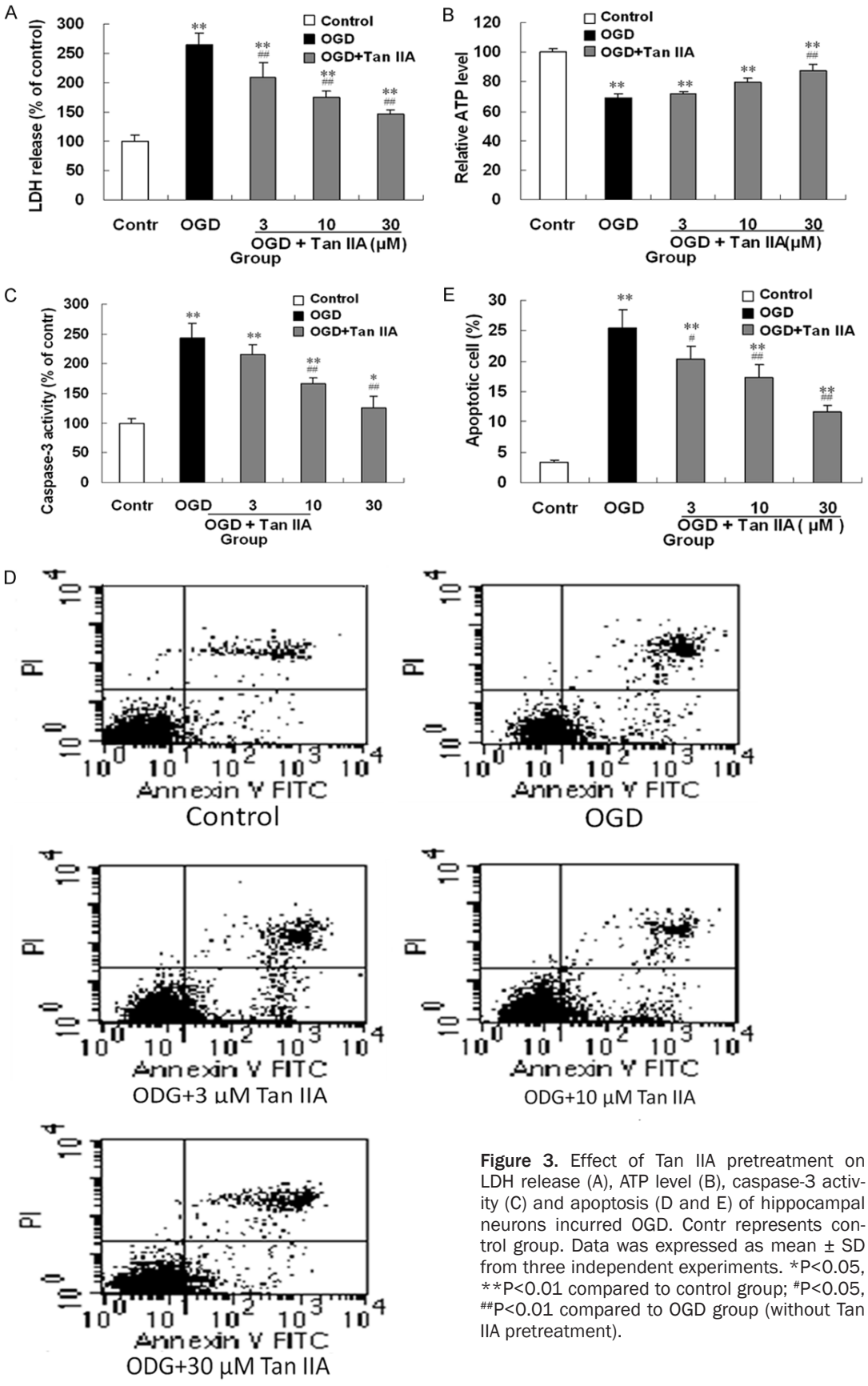

Figure 3. Effect of Tan IIA pretreatment on LDH release (A), ATP level (B), caspase-3 activity (C) and apoptosis (D and E) of hippocampal neurons incurred OGD. Contr represents control group. Data was expressed as mean ± SD from three independent experiments. *P<0.05, **P<0.01 compared to control group; #P<0.05, ##P<0.01 compared to OGD group (without Tan IIA pretreatment).

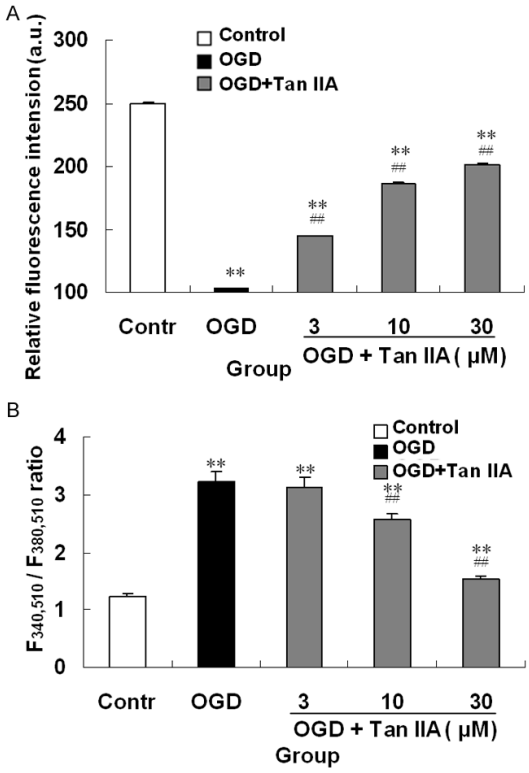

Figure 4. Effect of Tan IIA pretreatment on Δψ (A) and $[Ca^{2+}]_i$ (B) of hippocampal neurons incurred OGD. Contr represents control group. **P<0.01 compared to control group, ##P<0.01 compared to OGD group (without Tan IIA pretreatment).

Position: 768-850, Forward: CCTGGAGAAACCTGCCAAGTAT, Reverse: AGCCCAGGATGCCCTTTAGT). The arbitrary assigned Ct value was subtracted for UQCRFS1 to generate relative expression levels.

Following culture and treatment, equal amounts of hippocampal neurons were lysed in cell lysis buffer at 4°C for 30 min. Cell debris was removed by centrifugation at 15,000×g for 15 min. Equal amounts of proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were incubated in the blocking solution for 2 h at room temperature and incubated with monoclonal antibodies (1:1000 dilution, Abcam Biotechnology, Cambridge, UK) at 4°C overnight, followed by extensive washing with PBS twice and TBST twice. The membranes were then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and washed with TBST. GAPDH was used as an internal control. The immunoreactive proteins were detected using an ECL Western blotting detection system (Amersham Biosciences). The blots were subjected to densitometric analysis using ImageJ software (NIH Image analysis website http://rsb.info.nih.gov/ij/).

Statistical analysis

Calculation of statistical data was done by the statistical computer system GraphPad Instat 3. Analysis was performed using the statistical software package SPSS v18.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SD (standard deviation). Statistical analyses were performed using One-way analysis of variance (ANOVA) and unpaired two-tailed t-test. Differences at a *p*-value of less than 0.05 were considered statistically significant.

Results

Tan IIA attenuates OGD-induced cell death

Hippocampal neurons were cultured for 10-14 days and identified by immunocytochemistry with NSE monoclonal antibody (**Figure 1**). The percentage of NSE-positive neurons was more than 92%. The survival of neurons pretreated with Tan IIA was measured by MTT assay after the cells were OGD-ed for 6 h (**Figure 2**). Exposure of hippocampal neurons to OGD, neuronal survival was (61.5 ± 4.1)% as compared to control group (set 100%). After Tan IIA pretreatment at 3, 10, 30 and 100 μmol/L (mM), the cell survivals were (70.5 ± 2.1)%, (72.8 ± 4.4)%, (78.8 ± 3.4)% and (83.5 ± 3.1)% respectively and higher than that of only OGD-ed group (all p<0.05). Based on these results, we concluded that Tan IIA can attenuate OGD-induced hippocampal neuron death.

Tan IIA attenuates OGD-induced changes in LDH release, ATP level and apoptosis

After hippocampal neurons were incurred OGD for 6 h, culture supernatants and the cells were respectively collected for LDH concentration, ATP level and cell death detection. Cellular integrity damage was reflected by LDH level (**Figure 3A**). LDH levels in OGD group was (264.7 ± 19.3)% and higher than that of con-

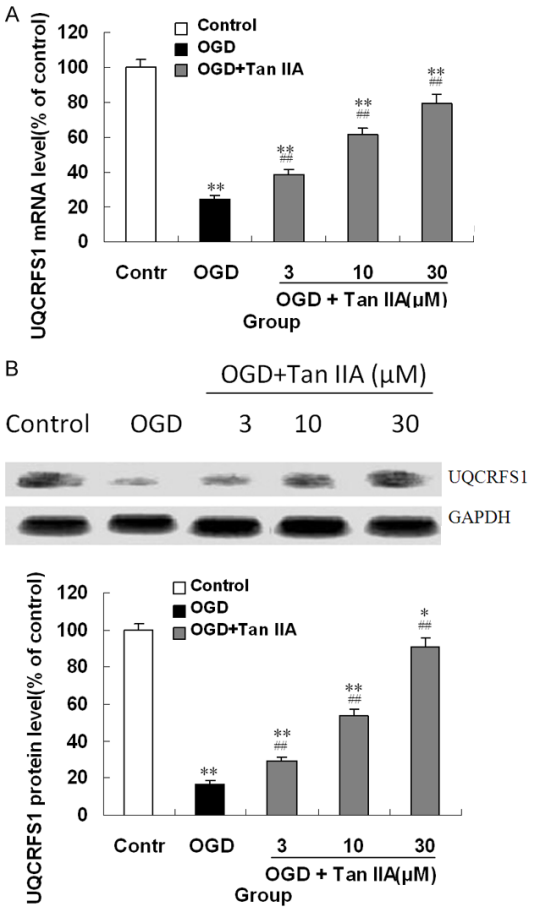

Figure 5. Effects of Tan IIA pretreatment on mRNA (A) and protein (B) expression of UQCRFS1 in cultured hippocampal neurons incurred OGD. Contr represents control group in which hippocampal neurons were neither pretreated with Tan IIA nor incurred OGD. The result was expressed as mean ± SD from three independent experiments. Multiple comparisons were performed by ANOVA. *P<0.05, **P<0.01 compared to control; ##P<0.01 compared to OGD-ed group without Tan IIA pretreatment.

trol group (not OGD-ed, set 100%) (p<0.01). Pretreatment of Tan IIA at 3, 10 and 30 mM reduced the LDH concentration by 20.9%, 34.2% and 44.8% respectively compared to the OGD group. ATP level in OGD group was lower than that of control group, and the effect was attenuated by Tan IIA pretreatment (**Figure 3B**). Caspase-3 activity detection (**Figure 3C**) and flow cytometry analysis (**Figure 3D**) showed that Tan IIA pretreatment attenuated elevation of caspase-3 activity and OGD-induced apoptosis of hippocampal neurons in a dose-dependent manner. These results indicate that Tan IIA pretreatment reduced OGD-induced cell death by ameliorating cellular integrity and decreasing apoptosis.

Tan IIA attenuates OGD-induced changes in mitochondrial membrane potential (Δψ)

Δψ, which is in proportion to the fluorescence intension of R123, was monitored by fluorospectrophotometer (**Figure 4A**). After exposure to OGD for 6 hours, Δψ of the hippocampal neurons was markedly decreased by 58.6% as compared to the control group. The OGD-induced Δψ reduction was rescued by Tan IIA pretreatment in a dose-dependent manner (**Figure 4**).

Tan IIA attenuates OGD-induced changes in $[Ca^{2+}]_i$

Intracellular Ca^{2+} homeostasis is required for maintenance of cell survival. In this study, $[Ca^{2+}]_i$ was detected by fluorospectrophotometer and indirectly reflected by the $F_{340,\ 510}/F_{380,\ 510}$ ratio of fura-2 AM (**Figure 4B**). After OGD induction for 6 hours, the $[Ca^{2+}]_i$ of the primary hippocampal neurons was elevated by approximately 2.6 times as compared to the control group. Pretreatment of Tan IIA at 10 and 30 μmol/L rescued the OGD-induced $[Ca^{2+}]_i$ loss. (**Figure 4**).

Tan IIA rescued the changes in UQCRFS1 expression after OGD-induction

After hippocampal neurons were incurred OGD for 6 h, both mRNA and protein level of UQCRFS1 were decreased in the cells as compared to the control group. Tan IIA pretreatment attenuated the effect of OGD on the cells in a concentration-dependent manner (**Figure 5**).

Discussion

Tan IIA was shown to prevent PC12 cells from apoptosis induced by serum-free culture conditions [24], neural progenitor cell line C17.2 from mitochondrial damage induced by 2,2'-azobis (2-amidino propane hydrochloride) and brain from damage by ligation of the right common carotid artery [22] or permanent middle cerebral artery occlusion [23]. In this study, we studied the effects of Tan IIA on primary cultured rat hippocampal neurons subjected to OGD. MTT assay demonstrated that Tan IIA pretreatment has neuroprotective effects on the OGD-exposed cells in a concentration-dependent manner. However, the underlying mechanism remains to be fully understood. As well known, neuronal function and

survival depend on a continuous supply of glucose and oxygen, used to generate ATP through glycolysis and mitochondrial respiration. Apoptosis is an active, energy-dependent process that requires ATP to initiate the molecular cascade [30]. Cells induced by integrity damage will release LDH (a sensitive indicator of cell damage, which is correlated with changes in neuronal morphology [31]) into cytoplasm and generate less ATP. After OGD, LDH efflux, intracellular calcium concentration and apoptotic percentage of the hippocampal neurons were increased, ATP level decreased, and intracellular caspase 3 activated in this study. The effects OGD-induced on the neurons were attenuated by Tan IIA pretreatment in a concentration-dependent manner. These data indicate that Tan IIA pretreatment can protect hippocampal neurons from apoptosis by reducing energy damage and ameliorating changes in intracellular calcium concentration, all these can be potentially beneficial for patients with ischemic diseases.

In all nucleated cells, mitochondria, as a central platform in the execution of diverse cellular events [32], are the principal generators of cellular ATP by oxidative phosphorylation, incorporating the electron-transferring respiratory chain (complexes I-IV) and the ATP synthase (complex V). Damaged mitochondria produce less ATP [33], resulting in excessive production of reactive oxygen species [34], which stimulate the release of cytochrome c from mitochondrial inter-membrane space to the cytosol [35] and decrease cell viability [36, 37]. UQCRFS1, the Rieske Fe-S protein (RISP), is a key subunit of the respiratory chain protein Ubiquinol Cytochrome c Reductase (UQCR, Complex III or Cytochrome bc1 complex) and one of the nuclear-encoded oxidative phosphorylation proteins. UQCRFS1 can be induced by serum [38] and generates an electrochemical potential coupled to ATP synthesis to transfer electrons from ubiquinol to cytochrome c. Recently, UQCRFS1/RISP knockdown in breast tumor cell line led to decreased mitochondrial membrane potential [39]. In the current study, we analyzed the mitochondrial membrane potential and UQCRFS 1 expression to reveal the underlying energy amelioration mechanisms of Tan IIA pretreatment in OGD-exposed hippocampal neurons. When the neurons were incurred OGD, decrease of mitochondrial membrane potential, ATP level and UQCRFS1 expression was observed. After pretreated with Tan IIA, these effects of OGD were ameliorated in the cells. These data indicate that Tan IIA pretreatment is involved in attenuating energy damage in hippocampal neurons induced by OGD-exposure. For potential cerebral ischemia population, Tan IIA administration might be beneficial in attenuating neurons damage and better prognosis when abrupt onset of hypoxia-ischemia cerebral disease.

In summary, the current study reveals that Tan IIA pretreatment rescues ODG-induced cells apoptosis and mitochondria dysfunction in hippocampal neurons by increasing UQCRFS1 expression. However, further *in vivo* studies are necessary to address whether Tan IIA pretreatment is effective in neuron protection and neurobehavioral consequences in animal models. Exploring these issues will help developing feasible strategy of Tan IIA pretreatment as a brain ischemic injury protective agent.

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Disclosure of conflict of interest

None.

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